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A mixture of peptides and sugars derived from plant cell walls increases plant defense responses to stress and attenuates ageing-associated molecular changes in cultured skin cells

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ABSTRACT

Small peptides and aminoacid derivatives have been extensively studied for their effect of inducing plant defense responses, and thus increasing plant tolerance to a wide range of abiotic stresses. Similarly to plants, these compounds can activate different signaling pathways in mammalian skin cells as well, leading to the up-regulation of anti-aging specific genes. This suggests the existence of analogous defense response mechanisms, well conserved both in plants and animal cells. In this article, we describe the preparation of a new mixture of peptides and sugars derived from the chemical and enzymatic digestion of plant cell wall glycoproteins. We investigate the multiple roles of this product as potential "biostimulator" to protect plants from abiotic stresses, and also as potential cosmeceutical. In particular, the molecular effects of the peptide/sugar mixture of inducing plant defense responsive genes and protecting cultured skin cells from oxidative burst damages were deeply evaluated.

1. Introduction

Accumulating evidence indicates that compounds that protect plants from environmental stresses, such as UV, drought, salinity, freezing and high temperature, produce similar protective effects on human skin, because they act via the activation of basic signal transduction mechanisms that are conserved in all eukariotic cells. Activation of defense response genes responsible for the protection and the repair of damage caused by stressing agents follows analogous mechanisms in plant and in human cells (Dalle-Donne et al., 2009; Scandalios, 2005).

Small peptides, hydroxyproline, betaine and other aminoacid derivatives have been extensively studied for their effect of inducing plant defense responses, and thus increasing plant tolerance to a wide range of abiotic stresses (Tuteja, 2007). Exogenous application of glycinebetaine, for example, improves growth and survival of a wide variety of plants under stress (Park et al., 2006; Cuin and Shabala, 2005). Also, the beneficial effect of proline against abiotic stresses and heavy metal toxicity has been well documented (see Sharma and Dietz, 2009 for review).

Similarly to plants, peptides and aminoacids, in particular proline, can activate different signaling pathways in human skin cells, leading to the up-regulation of specific ageing-associated genes and making cells more resistant to stress factors (Krishnan et al., 2008). Research has also demonstrated a beneficial effect of synthetic peptides and total protein extracts on wound healing (Bentley et al., 1990) and on the production of new collagen fibers (Katayama et al., 1993). However, most of these peptide and aminoacid mixtures used as ingredients in cosmetics contain either chemically synthesized peptides, or peptides obtained by the partial digestion of animal proteins, like collagen and elastin (Mokrejs et al., 2009; Langmaier et al., 2002). The use of peptides produced by hydrolysis of animal proteins always involves certain risks associated with the use of any product derived from animals. Contamination of the protein extracts by viruses or bioactive peptides of animal origin represents a serious potential problem for human health: one example is Bovine Spongiform Encephalopathy (BSE), which is thought to be related to the use of animal protein lysates (Yokoyama and Mohri, 2008).

To explore the use of natural plant-derived products we examined the effects of an aminoacid and peptide rich mixture obtained from plant cell cultures. Plant cell walls are particularly rich in proteins with high content of glycine and proline (Ringli et al., 2001), which are also the most abundant aminoacids

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in mammalian collagen. The cell walls of suspension-cultured plant cells are particularly rich in proteins as these proteins are induced during the de-differentiation process that occurs when pieces of plant tissue are put in culture and calluses are formed. The response of the tissue that has been put into culture is akin to a wound response, which is known to be accompanied by the enhanced expression of cell wall proteins (Chrispeels et al., 1974). The preparation that we obtained contained, besides the protein fraction derived from the partial hydrolysis of the extracellular matrix (cell wall) of the cultured cells, also a sugar fraction, derived from the glycoproteins present in the cell wall. Although plant cell wall proteins have a number of similarities with animal collagen, they are much safer to use in cosmetics because they are less likely to present risks to human health. Besides the peptides, also the sugars, mainly represented by hexoses, pentoses and oligosaccharides, enhance hydration, thus promoting the anti-ageing effect of the peptides on the skin cells. Even though the role of sugars in cosmetics has not clearly defined yet, it was shown that saccharides contained in a plant extract had beneficial effects on hydration and had an anti-flammatory effect on dermal cells (Eom et al., 2006).

We conducted a series of experiments using *Arabidopsis thaliana* to analyze the effect of a tobacco plant cell wall enzymatic digest consisting of a peptide/sugar mixture on the activation of plant defense genes. We also investigated its potential use as novel plant "biostimulator". Having obtained positive results, we investigated the efficacy of this cell wall extract as a potential cosmeceutical. Having demonstrated a protective effect of the peptide/sugar mixture against oxidative stress associated changes in cultured skin cells, we investigated whether this plant-derived mixture had additional beneficial effects on these cells. The study revealed that the preparation had multiple effects including upregulating genes involved in DNA protection and repair, and increasing collagen synthesis and stability.

2. Materials and methods

2.1. Preparation of the peptide/sugar mixture

Nicotiana tabacum BY2 (or *N. sylvestris*) cells were grown in liquid medium as described by Kato et al. (1972). Briefly, 50 ml of a dense culture was used as starting material for inoculating 1 l of culture medium, containing 30 g l^{-1} of saccharose, 4.4 g l^{-1} of Murashige/Skoog Basal Salt Mixture (MS salt, Sigma), 0.1 g l^{-1} of inositol, 1 mg l^{-1} of thiamine, 0.2 mg l^{-1} of 2,4D, and 0.18 g l^{-1} of KH_2PO_4 at pH 5.7. The culture was incubated at 27 °C, in the dark, under constant orbital stirring (110 rpm) and after 8 days the cells were collected and filtered through a layer of Miracloth fabric (Calbiochem). The drained cells (around 300 g) were then resuspended in 300 ml of PBS (NaCl 136 mM, KCl 2.7 mM, NaH_2PO_4 12 mM, KH_2PO_4 1.76 mM, pH 7.4), containing 1% of Triton X-100, and homogenized in a mortar. The resulting lysate was centrifuged at 10,000 rpm to precipitate the cell walls which were then washed with PBS to remove all the cytoplasmic components and the detergent. The pellet was first boiled for 20 min in 150 ml of EDTA 2 mM to remove starch and pectins, and then treated with 150 ml of HCl 0.1N at 90 °C for 1 h to hydrolyze the sugar bonds. After cooling the suspension, 1 mg ml^{-1} of pepsin was added and the cell walls were incubated at 37 °C for at least 24 h. After centrifugation at 10,000 rpm, the supernatant was collected, neutralized with NaOH and lyophylized. The powder obtained (around 150 mg) was dissolved in water and used in the assays at the required concentration.

2.2. Mass spectrometry and aminoacid analysis of the peptide/sugar mixture

For mass spectrometry, 3 μg of mixture in a volume of 10 μl was analyzed by applying a solvent gradient (TFA 0.05% in CH3CN) from 1% to 30% in 90 min, setting a flux of 200 μl min^{-1}. The parameters used for the analysis were: capillary temperature, 300; AGC Off Ion Time (ms), 5.0; Sheath Gas Flow (au), 65.0; and Aux/Sweep Gas Flow (au), 20.0. The peaks were detected in the spectrum of wavelength ranging from 200 to 320 nm.

To perform aminoacid analysis, 1 mg lyophylized aliquots of mixture were treated with 100 μl of HCl 6N, under vacuum at 150 °C for 1 h, to break all the peptidic bonds (Einarsson et al., 1987; Fountoulakis and Lahm, 1998). The analysis was conducted following a method of pre-column derivatization, using 9-fluorenilmetoxycarbonil chloride. The aminoacid derivatization was performed by dissolving the mixture in 250 μl of 0.5 M borate buffer, pH 7.70 and adding 250 μl of 15 mM Fmoc-Cl solution in acetone at room temperature. After 45 s, two extractions in n-esane were performed to remove the excess of reagent. Ten μl volume of the solution containing free aminoacids was analyzed by RP-HPLC, using the system HPLC Alliance (Waters) provided of quaternary pump and detector operating with flux of 1.25 ml min^{-1}, temperature 45 °C and wavelength from 200 to 400 nm. The aminoacid ratios and their total amounts were calculated comparing the peak areas in the chromatogram at 263 nm wavelength. A solution of all the aminoacids, used as standard, was commercially available from Pierce, and contained equimolar concentrations of all the aminoacids (2.5 μmol ml^{-1} in 0.1 N HCl, except L-cysteine, at a concentration of 1.25 μmol ml^{-1}).

2.3. Plant treatments with peptide/sugar mixture

Arabidopsis thaliana (L.), strain Columbia (Col-0) was grown on soil in a growth-chamber under 16 h of light/8 h of dark photoperiod for 3–4 weeks. Seedlings were sprayed with 0.25% Murashige and Skoog salt base (pH 5.7) buffer containing the compounds to test at concentrations from 12 to 300 ppm. Leaves from three different plants for each treatment were collected, 16 h later pooled together and used in RT-PCR experiments. Four-week old plants of *Cucumis sativus* L. were sprayed with the sugar/peptide mixture at a concentration of 300 ppm and after 6 h treated with the oxidative agent, Paraquat (1,1′-dimethyl-4,4′-bipyridinium methylviologen, Sigma) at 35 mg l^{-1}. Every 24 h after the stress, plants were observed to detect eventual damages and necrosis on the leaf surface.

2.4. Semi-quantitative reverse transcriptase/polymerase chain reaction (RT/PCR)

Total RNA was extracted from plant tissues of *A. thaliana* (see Section 2.3) or mammalian cells (NIH-3T3 or HaCaT cells, see Sections 2.6 and 2.7) with the GenElute Mammalian Total RNA Purification Kit (Sigma) according to the manufacturer's instructions and treated with RNAse-free DNAseI at 37 °C for 30 min to eliminate any contaminating genomic DNA. The first strand cDNA was synthesized from 1 to 2 μg using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas).

Multiplex RT-PCR was performed using specific primers and the QuantumRNA™ 18S internal standard (Ambion) according to the manufacturer's instructions. The QuantumRNA™ kit contains primers to amplify 18S rRNA along with competimers that reduce the amplified 18S rRNA product within the range to allow it to be used as endogenous standard.

The genes of interest were amplified with the following primers:

PR1-F, 5′gtagctcttgtaggtgctct3′ and PR1-R, 5′catcctgcatatgatgctcc3′; PDH-F, 5′tgcttgtctatggcgtcgaa3′ and PDH-R, 5′ttacgcaatcccggcgatta3′; GSTF7-F, 5′cggtcacccagcttccacag3′ and GSTF7-R, 5′cactgacatgtggacgctcg3′; SAG12-F, 5′gcacatcgagtggatgactaa3′ and SAG12-R, 5′gggacatcctcataacctgta3′; OXI1-F, 5′gctagagggagatgagaaac3′ and OXI1-R, 5′gcgtaaaatctgataatctc3′; CAT3-F, 5′gtgctcaccacaacaatcacc3′ and CAT3-R, 5′ctttttgccggatttaccgcc3′; Gadd45α-F, 5′gactttggaaggaattctcggc3′and Gadd45α-R, 5′tgatccatgtagcgactttcc3′; Sirt1-F, 5′ atacccccatgaagtgcctc3′and Sirt1-R, 5′cgtcatcttcagagtcgta3′; Sirt6-F, 5′atgtcggtgaattacgcg3′and Sirt6-R, 5′actgcgtcttacacttgg3′; ColI-F, 5′gctaaaggtgccaatggtgctc3′and ColI-R, 5′ttttgccatcaggaccagggct3′; ColIII-F, 5′atgtctggaagcagaaccatg3′and ColIII-R, 5′ttgatacctggaggtccaggca3′; MMP1-F, 5′tttgggctgaaagtgactgg3′ and MMP1-R, 5′gggataacctggatccatag3′; MMP3-F, 5′ccattggatggagctgcaag3′ and MMP3-R, 5′aagtgggcatctccattaatcc3′; MMP9-F, 5′gtaccgctatggttacactcg3′ and MMP9-R, 5′tactgcaccagggcaagccg3′.

The amplification reactions were performed with the following general scheme: 2 min at 94 °C followed by 35 cycles of 94 °C for 30 s, annealing temperature (specific for each gene) for 30 s, and 72 °C for 30–60 s, with a 10 min final extension at 72 °C.

The PCR products were loaded on 1.5% agarose gel, and the amplification bands were visualized and quantified with the Geliance 200 Imaging system (PerkinElmer). The amplification band corresponding to the gene analyzed was normalized to the amplification band corresponding to the 18S. The values obtained were finally converted into percentage values by considering the measure of the untreated controls as 100%. All the semi-quantitative RT-PCRs were repeated three times and a representative result was displayed for each gene assay.

2.5. TAC and ORAC assays

Both the assays were used to measure the total anti-oxidant power of the peptide/sugar mixture by “in vitro” reactions. The Total Anti-oxidant Capacity (TAC) assay is based on a redox reaction between a test compound and copper II, Cu(II). If a test compound has got reducing power, the Cu(II) is readily converted into Cu(I) which can be monitored by the addition of a chromophore, Bathocuproine (BATO). Fifty μl of peptide/sugar mixture solution in water (at concentrations ranging from 0.025% to 0.1%, w/v) was aliquoted into a 96-well plate, 50 μl of a BATO solution (360 μM) was added to each well and the background absorbance at 490 nm was measured, then 25 μl of 100 μM $CuSO_4$ solution was added to each sample and incubated at room temperature for 30 min. At the end of the incubation time, the absorbance at 490 nm was measured by a plate reader (Victor3, PerkinElmer). As reference standard, scalar dilutions of CuCl were used, ranging from 10 to 0.15 mM.

Oxygen Radical Absorbance Capacity (ORAC) assay is based on the ability of a test compound to inhibit the oxidation of a fluorophore, generally fluorescein, by a potent oxidant, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) (Huang et al., 2002). Twenty-five μl of peptide/sugar mixture dilutions in phosphate buffer 75 mM, pH 7.4 (at concentrations ranging from 0.01% to 0.25%, w/v) was aliquoted into 96-well plate and 150 μl of fluorescein solution (8.5 nM in phosphate buffer) was added to each sample. After incubation at 37 °C for 15 min, 25 μl of AAPH solution (153 mM in phosphate buffer) was pipetted into each well and the progress of the reaction monitored at 535 nm, using a fluorescence multi-well reader. The fluorescence was measured every minute for 40 min. Anti-oxidant power of the mixture was calculated according to the method described by Huang et al. (2002). The net area under curve (AUC) of the samples and standards (represented by different dilutions of Trolox) was calculated. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the two measurements for each concentration. Net AUC was obtained by subtracting the AUC of the blank from that of the sample or the standard. ORAC values of the samples were expressed as mmole of Trolox equivalents per liter.

2.6. Reactive Oxygen Species (ROS) assay

1.5×10^4 NIH-3T3 cells (murine embryonic fibroblasts, ECACC, Salisbury, UK), maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FCS under 5% CO_2 at 37 °C, were seeded in 96-well plates and grown for 20 h to let them attach to the bottom of the well. The cells were then incubated with different concentrations of peptide/sugar mixture or ascorbate 250 μM, used as positive control, for 2 h. At the end, the cells were washed in PBS and the cell auto-fluorescence value (background) measured at the plate reader, using 490 nm as excitation and 535 nm as emission wavelength. The cells were then incubated with the dye CM-DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, Invitrogen) at 37 °C for 30 min. After an additional wash in PBS, the cells were treated with H_2O_2 150 μM and incubated for 30 min. The fluorescence of the samples was thus measured at 535 nm (excitation 490 nm), using the instrument EnVision (PerkinElmer), and from each measure the cell auto-fluorescence value was subtracted. The values obtained from stressed cells (with or without pre-treatment with the compounds) were expressed as percentage to the unstressed control, set as 100%.

2.7. Mammalian cell treatment for expression analysis

In order to analyze the expression of GADD45α and SIRT genes, NIH-3T3 cells, seeded at a density of 1.5×10^5 per well, were grown in 6-well plates. After 20 h, the cells were incubated with 1.2 and 3.6 μg/ml of peptide/sugar mixture, with ascorbate or *Mirtus communis* extract for 2 h. At the end, H_2O_2 at a final concentration of 150 μM was added to each well and after 6 h (for GADD analysis) or 3 h (for SIRT analysis) the samples collected and processed for RNA extraction.

The expression of MMP genes was analyzed in HaCaT cells (human keratinocytes, ECACC, Salisbury, UK), maintained in Dulbecco’s modified Eagle’s medium (DMEM) with FBS under 5% CO_2 at 37 °C and seeded at a density of 1.5×10^5 in 6-well plates. After 20 h, the cells were completely attached to the bottom of the well and thus incubated with 1.2 and 3.6 μg ml^{-1} of peptide/sugar mixture for 2 h. At the end 900 μM nickel was added to each well and after 3 h the samples collected and processed for RNA extraction.

2.8. Single cell electrophoresis (comet assay)

NIH3T3 cells, maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FCS under 5% CO_2 at 37 °C, were plated at a density of 1.5×10^5 per well in 6-well plates. After 3 h incubation with the peptide/sugar mixture, cells were treated with H_2O_2 150 μM for 2.5 h and were detached from the plate using 1 ml/well of Sigma non-enzymatic solution. Cells were transferred to eppitubes, spun at 1.5 k and washed once with PBS1X. The cell pellet was resuspended in 10–20 μl of PBS, depending on the amount of cells recovered. Eighty–ninety μl volume of Low Melting Point Agarose (LMPA) 0.5% in PBS and equilibrated at 37 °C was added to each tube, and the solution immediately dropped onto a Normal Melting Agarose (NMA) pre-coated slide. Coverslip slides were placed on the top, without squeezing the cells, and the slides were put on a tray in the fridge until the agarose layer hardens (10–15 min). Coverslips were then gently slided off without scraping the agarose layer containing the cells. The slides were placed in cold Lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma Base pH 8.5, 1% TritonX100) for at least 2 h at 4 °C. Slides are gently removed from

Lysis solution and are placed in an electrophoresis tank, filled with cold Electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13). Slides are left in alkaline buffer for 10 min to allow DNA unwinding and the expression of alkali-labile damage, and then power supply is turned on to 24 V. Slides were electrophoresed for 20 min and at the end placed in cold Neutralization buffer (0.4 M Tris–HCl, pH 7.5) for at least 10 min. After drying the slides, cells are stained with a 10 $\mu g\,ml^{-1}$ solution of ethidium bromide, covered with a coverslip and scored at a fluorescence microscope.

2.9. Collagen expression and synthesis analysis

For collagen expression analysis, NIH-3T3 cells, seeded at a density of 1.5×10^5 per well, were grown in 6-well plates for 20 h. The cells were then incubated with different concentrations of peptide/sugar mixture or ascorbate 250 μM for 6 h. Afterwards, the samples were collected and processed for RNA extraction, as described above.

To measure newly synthesized collagen by ELISA, NIH-3T3 cells were seeded in 96-well plates at a density of 8×10^3 per well, and treated with the reagents for 24 h. After washing in PBS, cells were fixed in formaldehyde 4% and incubated with primary goat monoclonal antibodies, raised against collagen type I and type III (Santa Cruz Biotechnology), dissolved in PBS containing BSA 1%. Two hours later, samples were washed in PBS three times, and incubated with anti-goat secondary antibody, HRP labeled. The amount of new collagen produced by the cells was measured by a colorimetric reaction using a 0.5 $mg\,ml^{-1}$ solution of OPD, 0,012% H_2O_2 in citrate buffer 50 mM. The plate was incubated at room temperature for 30 min. At the end, the absorbance at 490 nm was measured by a plate reader (Victor3, PerkinElmer).

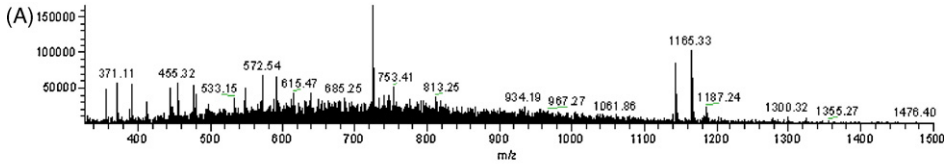
3. Results and discussion

3.1. Peptide/sugar mixture characterization

To obtain the peptides and the sugars from plant cells, we made cell wall preparations from liquid cultures of *Nicotiana* sp., using a modification of the Cho and Chrispeels (1976) procedure to reduce the number of steps. After the cell walls were isolated from the total homogenate, they were extensively washed to remove contaminating cytoplasmic components and to ensure that only proteins and glycoproteins covalently attached to the cell walls contributed to the mixture of peptides and carbohydrates obtained by chemical and enzymatic treatment of the cell wall preparation. The peptide/sugar mixture obtained was analyzed by mass spectrometry to determine the range of MW of the components (Fig. 1A). The analysis showed that most of the compounds present in the mixture have a MW ranging from 500 to 900 Da, thus both the peptides and the sugars are represented by oligomers of 3–8 units. Further analysis of the fragmentation spectra of some peaks of peptides indicated the presence of internal sequences rich in proline or Ile/Leu/Hyp. The relative percentages of the aminoacids present in the mixture, reported in Fig. 1B, were determined by a pre-column derivatization method, based on the use of 9-fluorenilmetossicarbonyl chloride (as described in Section 2). Glycine and proline, 16% and 8.5% respectively, were the most abundant aminoacids in the mixture, which is also the case in animal collagen. The total content of sugars was determined by the phenol/sulphuric acid method, described in Buysse and Merckx (1993), and was about 8 mg per mg of total protein extracted: this means that the sugar fraction and the protein one represent nearly 88% and 12%, respectively, of the total mixture. The sugar fraction is mostly composed of hexoses, such as galactose, glucose, mannose, xylose, and pentoses, such as arabinose, confirming other findings (Shpak et al., 1999; Popper, 2008).

3.2. Effect of the peptide/sugar mixture on plant defense response

To study the effect of the peptide/sugar mixture on plant defense gene expression, we sprayed 3-week-old *Arabidopsis* plants with different amounts of the mixture (ranging from 12 to 300 ppm) and analyzed the expression of stress marker genes by RT-PCR. *A. thaliana* is a model plant and thus used in most of the studies involving gene expression analysis. For this type of analysis we first chose three genes which represented excellent molecular markers for quantifying the defense response of the plant to different abiotic stresses. The genes analyzed were proline dehydrogenase (PDH)



(B)	D+N	2.93
	HYP	3.1
	S	4.7
	E+Q	4.8
	R	7.6
	T	6.1
	G	16
	A	5.9
	Y	5
	P	8.5
	V	7.2
	F	4.7
	C	0
	I	5.1
	L	6.8
	H	3.7
	K	7.6
		100.00%

Fig. 1. Mass spectrometry analysis and aminoacid composition of the peptide/sugar mixture. (A) Molecular weight distribution of all the compounds present in the mixture: the region with the highest peak density ranges from 500 to 900 Da. (B) Each aminoacid is expressed as percentage related to all the other aminoacids present. The most abundant is glycine, which is 16% of all aminoacids.

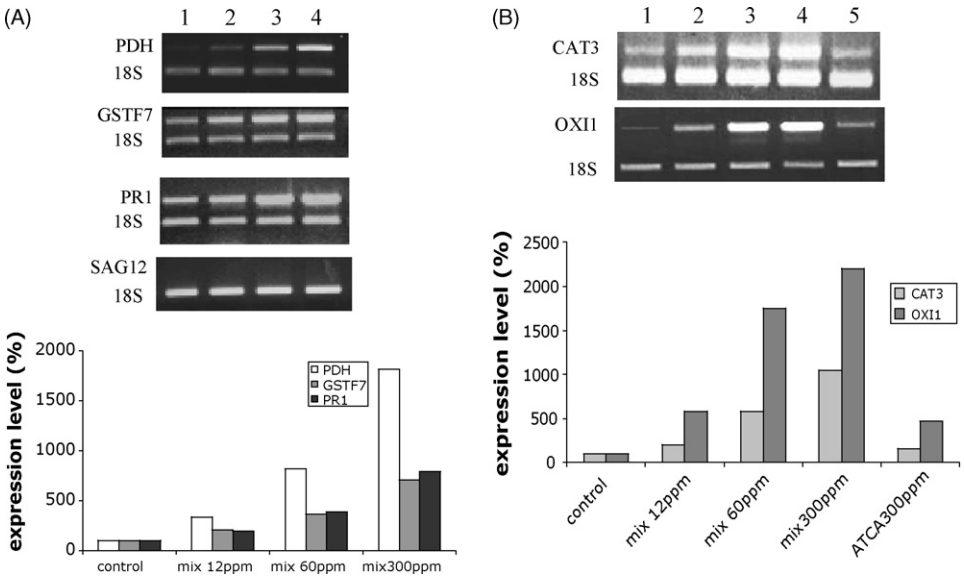


Fig. 2. RT-PCR analysis of gene expression in plants treated with different amounts of peptide/sugar mixture. The pictures show the amplification bands of the genes PDH, GSTF7, PR1 and SAG12 (A) and of the genes CAT3 and OXI1 (B). The amplified bands were quantified using Geliance 200 Imaging software and the values obtained normalized to the reference standard 18S. All the values were reported in the graphs below the pictures and expressed as percentage to the control, set as 100%. The expression of SAG12 was not detectable so the corresponding column was not reported in the graph. Lane1, control; lane2, 12 ppm of mixture; lane3, 60 ppm of mixture; lane4, 300 ppm of mixture; and lane5, 300 ppm of ATCA.

(Kiyosue et al., 1996), glutathione S-transferase F7 (GSTF7) (Sappl et al., 2004) and the pathogenesis-related protein 1 (PR1) (Martin et al., 2003). While the first two genes are involved in the defense to abiotic stresses mediated by proline, the third gene has been described as more specific marker of biotic-type stresses, involving salicylic acid as a mediator (Shah, 2003). However, recently it was found that certain PR family members are also induced in response to different abiotic stresses and chemical treatments (Seo et al., 2008). As shown in Fig. 2A, treatment with different amounts of the mixture led to significant induction of all three genes. Moreover, the level of gene induction was proportional to the dose of the mixture sprayed, confirming a specific protective effect on the plants induced by the peptide/sugar mixture. The amount of expressed gene was also quantified by an Imaging system software and the values obtained were expressed as percentages and reported in the histogram below. Plants that were sprayed with the mixture looked healthy, but to make sure that the doses of the mixture we used did not have any negative effect on the plants, we measured the expression level of a senescence marker gene, SAG12, which was also involved in the hypersensitive response after chemical treatments with toxic substances (Miller et al., 1999). Similarly to the untreated control, the expression level of SAG12 was null in all the plants sprayed with the different amounts of peptide/sugar mixture (Fig. 2A).

The induction of defense response of plants produced by the mixture was further evaluated by analyzing other genes which are more specifically involved in the oxidative stress response of plants. We chose CAT3, a gene encoding catalase, an enzyme involved in the degradation of hydrogen peroxide (Guan and Scandalios, 2000), and OXI1, one of the key elements of the transduction pathway linking oxidative burst signals to diverse downstream responses (Rentel et al., 2004). The pictures and the histogram in Fig. 2B show the level of activation for each gene, in the untreated and the treated plants. Also for the genes CAT3 and OXI1, the mixture produced a significant effect of activation, suggesting a more specific role of protection against oxidative stress damage. In the same experiment other plants were treated with the proline analog acetyl-thiazolidine-4-carboxylic acid (ATCA), known to be an inducer of proline-mediated abiotic stress responses (Elthon and Stewart, 1984). The levels of activation produced by the peptide/sugar mixture were much higher than those produced by ATCA (10 times versus 2 times for CAT3, and over 20 times versus 5 times for OXI1 gene).

The mitigation of the effects normally accompanying abiotic stress induced by the mixture was also analyzed on cucumber (*Cucumis sativus* L.), which is a large leaf plant and thus more commonly used to see the damage of oxidative stress on the leaf surface and to quantify the protection of an agricultural product. After 6 h treatment with 300 ppm solution of peptide/sugar mixture, the plants were sprayed with 35 mg/L of Paraquat, a free radical-generating herbicide which mimics strong oxidative burst in the plant tissues (Silverman et al., 2005). Every 24 h after the stress, the plants were observed to detect eventual damages on the leaves, and compared to untreated plants (control) and plants treated only with Paraquat. The plants pre-treated with the mixture showed a significant reduction of the necrotic spots caused by the oxidative agent, and developed more similarly to the untreated control plants (Fig. 3). All the results obtained clearly indicate that the cell wall extracted mixture of peptides and sugars had a significant effect of increasing plant tolerance to oxidative stress, by upregulating key genes involved in the defense response mechanisms.

3.3. *Anti-oxidant effect of the peptide/sugar mixture on skin cells*

To evaluate the potential of the peptide/sugar mixture as a cosmeceutical, we conducted a series of experiments to measure the total anti-oxidant power of the mixture both “in vitro” and in cultured skin-derived NIH-3T3 cells.

The anti-oxidant activity of a compound or a mixture of compounds depends on its total reducing capacity, on its ability to penetrate into the cells, and on its capacity to trigger a signal cascade leading to the production of endogenous anti-oxidant compounds in the cells. “In vitro” assays measure the reducing potential of the test material; however they do not provide any information on the anti-oxidant induction in living cells. Assays on cells, like ROS assay, measure the amount of reactive oxygen species (ROS)

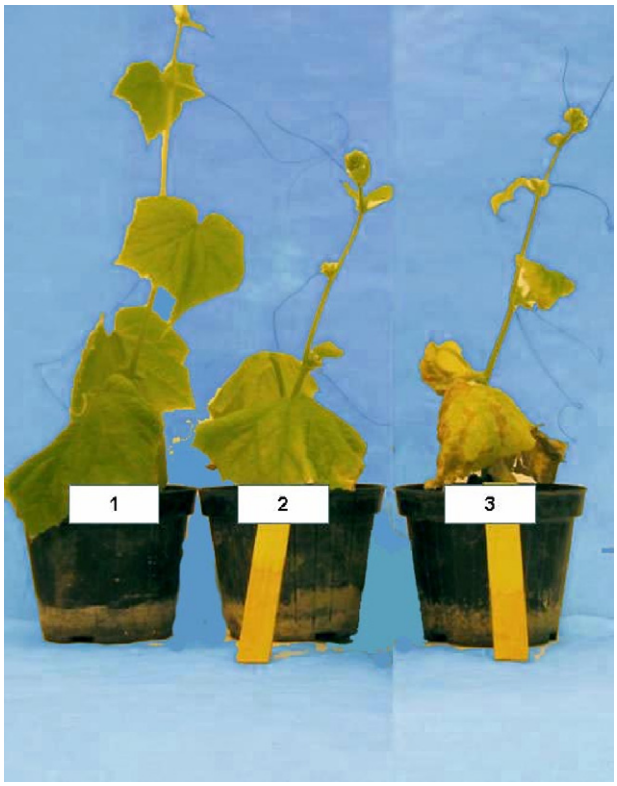


Fig. 3. Effect of the peptide/sugar mixture on stressed cucumber plants. Four-week old plants, sprayed with 300 ppm of peptide/sugar mixture and then treated with the oxidative agent Paraquat (sample 2), were compared to untreated plants (sample 1) and plants treated only with Paraquat (sample 3).

present in the cells; moreover, it can give clues about the protective effect of the test material under stress conditions. Both kinds of assays were used to evaluate the anti-oxidant activity of the peptide/sugar mixture.

As "in vitro" assays, we performed both TAC (Total Anti-oxidant capacity) and ORAC (Oxygen Radical Absorbance Capacity), described in Section 2. In the TAC, different concentrations of the mixture (ranging from 0.1% to 0.25% of dry, w/v) were mixed with 100 mM of Cu(II) and BATO, and after 30 min the amount of Cu(I) produced was measured at 490 nm. In the ORAC assay, the different dilutions of the mixture were mixed with fluorescein and the reaction started by the addition of AAPH. The values of reducing capacity were expressed as total moles of Cu(I) produced for

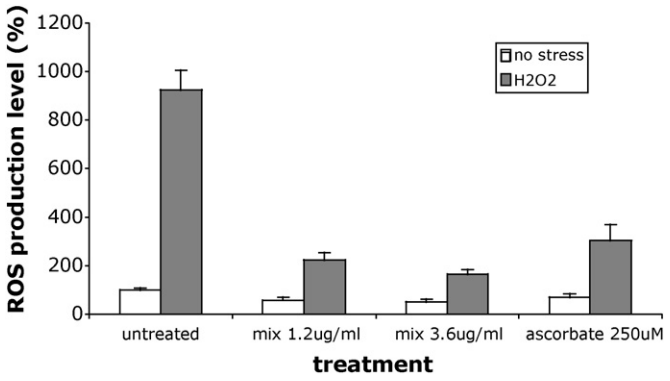


Fig. 5. Production of Reactive Oxygen Species (ROS) in NIH-3T3 cells treated with different amounts of mixture and with ascorbate. After treatment, the cells were incubated with and without H_2O_2 150 μM for 2 h and the amount of ROS produced in the cells measured by the fluorescent dye DCFDA, as described in Section 2. The values are means of 5 independent measures obtained from one representative experiment.

TAC assay, and as μmol of Trolox L^{-1} for ORAC assay. From the results shown in Fig. 4, it is possible to calculate the total reducing capacity of the mixture: it was 7.2 mmol L^{-1} of Cu(I) produced from every gram of product, and 1.57×10^2 μmol L^{-1} of Trolox per gram, values comparable to those of other anti-oxidant compounds or extracts used in cosmetics (Yildiz et al., 2008; Huang et al., 2002).

To evaluate the protective anti-oxidant effect of the peptide/sugar mixture on the cells, we treated mammalian NIH-3T3 fibroblasts, with two different concentrations of the mixture before being stressed by H_2O_2. Before performing the cell assays, the IC50 of the mixture was calculated by cytotoxicity assays, performed both on NIH-3T3 fibroblasts and HaCaT keratinocytes. The concentrations of the mixture we chose to use in all the tests were much lower than the IC50 value, which was 1.1 mg per ml. A reduction of ROS production was observed in both the samples pretreated with the mixture (1.2 and 3.6 μg/ml) compared to the untreated sample (Fig. 5). Moreover, a reduction of about 85% was observed in the sample treated with 3.6 μg/ml of mixture after induction of ROS by using H_2O_2. The reduction of ROS produced by the mixture treatment was even higher than that produced by the well-known anti-oxidant agent, ascorbate, used at a concentration of 250 μM (44 μg per ml). The attenuation of the H_2O_2-induced oxidative burst produced by the peptides and sugars that constitute the mixture can be explained both by a direct interaction with the stressing agent (the aminoacids and the sugars can chemically react with H_2O_2, neutralizing its oxidizing action on the cellular components)

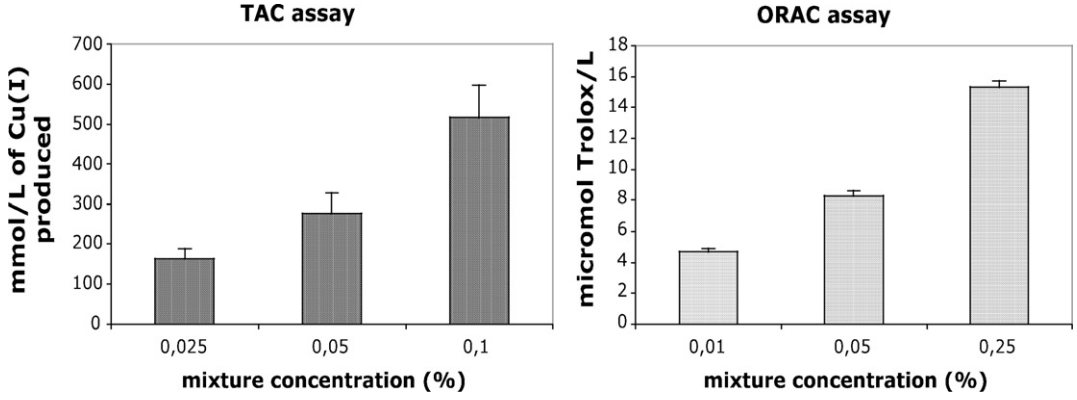


Fig. 4. Total anti-oxidant power of the peptide/sugar mixture measured by TAC and ORAC assays. Three different concentrations of the mixture (expressed as %, w/v) were analyzed for their anti-oxidant power by using the Total Anti-oxidant Capacity (TAC) assay and the Oxygen Radical Absorbing Capacity (ORAC) assay. The values reported in the graphs are averages of three independent measures, derived from three different experiments, and the error bars represent standard deviations.

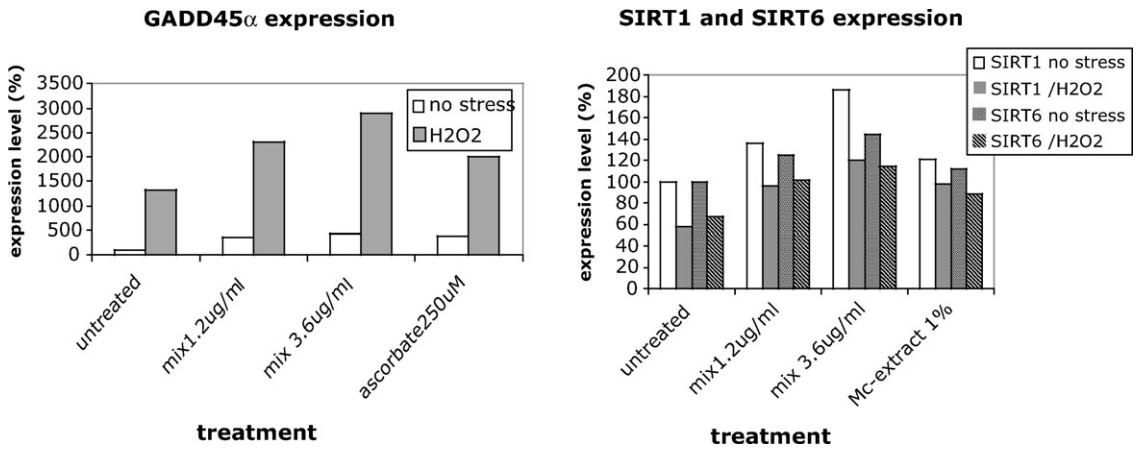


Fig. 6. RT-PCR analysis of gene expression in NIH-3T3 cells treated with two different concentrations of peptide/sugar mixture and with 250 μM ascorbate or 1% *Mirtus communis* leaf extract (Mc-extract). Cell samples were treated with the mixture or the compounds indicated, then incubated for 2 h with H_2O_2 and the mRNA level of the genes GADD45α or SIRT1 and SIRT6 analyzed by RT-PCR.

and by activation of defense response signaling pathways in the cells.

3.4. Study of the effect of the peptide/sugar mixture on gene expression

On the basis of the anti-oxidant proprieties shown by the mixture on the cells, we determined whether this mixture could have any effect on the expression of genes involved in DNA protection. Among the genes, we selected GADD45α, a member of GADD45 family that includes proteins with key roles in DNA repair (Takekawa and Saito, 1998), SIRT-1 and SIRT-6, both belonging to the family of sirtuins, proteins with deacetylase and ADP-ribosyl transferase activities NAD-dependent (Lavu et al., 2008). For GADD45α expression analysis, fibroblasts were treated with 150 μM H_2O_2 alone, H_2O_2 in the presence of two different concentrations of peptide/sugar mixture, and H_2O_2 in the presence of ascorbate, used as positive control. After 6 h of treatment, cells were collected and the expression of GADD45α analyzed by RT-PCR. As shown in Fig. 6, both the treatment with the mixture and with ascorbate produced a significant increase in GADD45α expression in the absence of H_2O_2. Even in the presence of H_2O_2, which causes an increase of GADD45α expression due to genomic DNA damages (Liebermann and Hoffman, 2007), the mixture increases the expression of this gene, suggesting a mechanism of protection against DNA damages even in the presence of the oxidative stressing agent.

In analogous experiments, we analyzed the expression of SIRT-1 and SIRT-6 by RT-PCR in cells treated with different concentrations of peptide/sugar mixture, in the absence and presence of H_2O_2. The H_2O_2 treatment produced an inhibition of sirtuin expression, but this effect was significantly attenuated by the presence of the mixture, suggesting a mechanism of protection against oxidative burst (Fig. 6). Also in the absence of H_2O_2, the mixture produced a significant induction of SIRT-1 and SIRT-6 expression.

The effect of a stressing agent, such as H_2O_2, on cell genomic DNA can be observed by the "single cell electrophoresis assay"

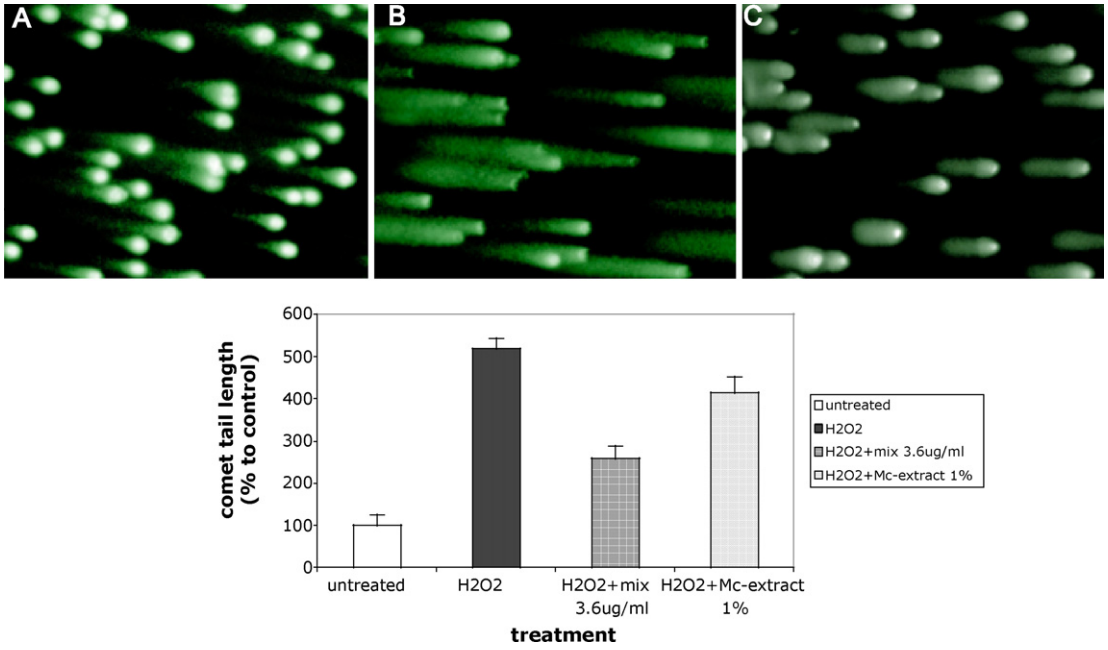


Fig. 7. Comet assay. The pictures show the damages to cell nuclear DNA produced by H_2O_2 treatment (B and C) compared to an untreated control (A). Cells in the panel C were previously incubated with 3.6 μg/ml of peptide/sugar mixture for 3 h. Each value was calculated in microns as average of 100 different measures, and then expressed as percentage to the untreated control, arbitrarily set as 100%.

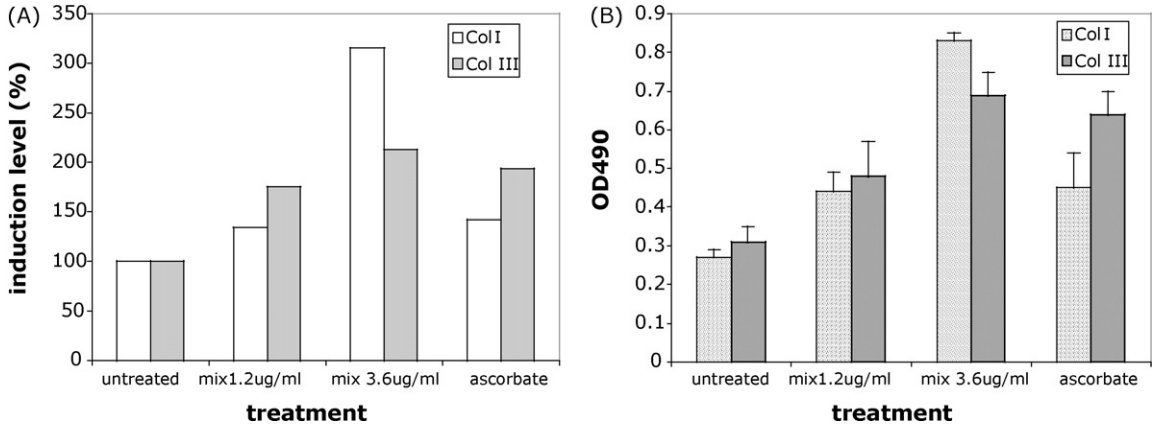


Fig. 8. Collagen type I (ColI) and type III (ColIII) expressions and their synthesis in cells treated with the peptide/sugar mixture. Cells were treated with the indicated concentrations of the mixture and with ascorbate, known as collagen inducer. After 6 h, RNA was extracted and ColI and ColIII expressions were analyzed by RT-PCR (A). The synthesis of new collagen (pro-collagen) was also measured after 16 h by ELISA (B).

(comet assay). This assay is based on the direct measure of DNA fragmentation, an index of the entirety of DNA damages produced by the oxidative burst. As shown in Fig. 7, the treatment with the peptide/sugar mixture produced a clear protection of genomic DNA from damages caused by H_2O_2. The length of the nucleus comet, index of DNA fragmentation, is significantly reduced in the cells treated with the mixture compared to the samples treated with only H_2O_2. A sample of cells was treated with another plant product (Mc-extract), known to contain compounds with protective effects on the cells from DNA-induced damages (Hayder et al., 2008). This product is a total hydrosoluble *M. communis* leaf extract at a stock concentration of 35 g l^{-1}, and already used as active ingredient in cosmetic formulations (INCI name: Water and Hydrolyzed Myrtus communis leaf extract, CAS no. 7732-18-5/84082-67-7). In the assay, the stock was diluted in water 100 times (1% final, corresponding to 0.35 mg ml^{-1}) as suggested by the manufacturer (Silab, Brive Cedex, France). The graph reported in Fig. 7 shows that the reduction of tail length produced by the peptide/sugar mixture is significantly higher than that produced by the Mc-extract (50% versus 20%), suggesting a better DNA protective capacity of the mixture than that provided by a commercially available skin care product.

3.5. Effect of the peptide/sugar mixture on collagen synthesis and degradation

Since many of the plant extracts used in cosmetics, besides their role as anti-aging products, have also been studied for their effects in inducing the synthesis of new extracellular matrix proteins (Knott et al., 2008), we analyzed whether the peptide/sugar mixture had any effect also on the collagen gene expression and collagen stability. To evaluate the production of new collagen by cultured fibroblasts, cells were incubated with 2 different concentrations of the mixture, and with ascorbate 250 μM, known as inducer of new collagen synthesis (Amano et al., 2006), and then collagen type I and type III gene expressions were analyzed. As reported in Fig. 8, the mixture produced a significant effect of increasing both collagen I and collagen III gene expressions: this increase was about 200% in the samples treated with 3.6 μg ml^{-1} of mixture, significantly higher than that produced by ascorbate. The level of pro-collagen in the cells was measured by ELISA, using monoclonal specific antibodies raised against pro-collagen type I and type III. The same figure shows that the levels of both pro-collagen I and pro-collagen III were higher after the treatment with the mixture, and the amounts of pro-collagen in the cells incubated with the highest dose of mixture was 2–3 times higher than that in the untreated cells.

In another series of experiments, we studied potential collagen stability following the treatment with the peptide/sugar mixture by analyzing the expression of MetalloProteinases genes. The Matrix MetalloProteinases (MMPs) are zinc-dependent endopeptidases, capable of degrading all kinds of extracellular matrix proteins, including triple-helical fibrillar collagens (Burrage et al., 2006). All three kinds of proteinases can be generally called collagenases, since all three are able to hydrolyze different types of collagens. Since it has been shown that nickel exposure results in prolonged induction of some collagenases (Perfetto et al., 2007), we studied the effect of the peptide/sugar mixture on nickel stressed HaCaT cells (keratinocytes), and analyzed the expression of collagenase-1 (MMP-1), stromelysin-1 (MMP-3) and gelatinase-B (MMP-9). After the treatment with the mixture, cells were incubated with 900 μM nickel to induce MMP expression. At the end, the expression of the collagenases was analyzed by RT-PCR. As shown in Fig. 9, the pre-treatment of the cells with the mixture reduced the induction of all the proteinase genes, and in the presence of nickel the mixture neutralized the up-regulation of MMP genes produced by the metal. This result suggests that the product, besides its effect of inducing genes for new collagen synthesis, has also an effect of inhibiting the expression of genes

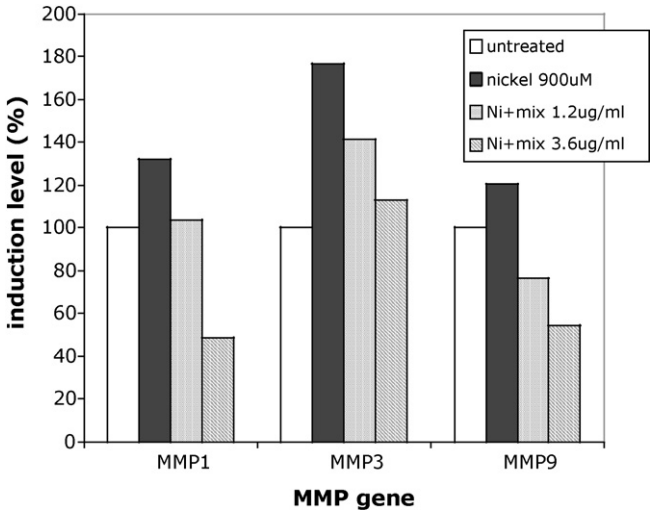


Fig. 9. MMP gene expression level in cells treated with nickel and the peptide/sugar mixture. HaCaT cells were treated with the indicated concentrations of mixture and then incubated in the presence of nickel 900 μM. RNA was extracted and the expression level of the proteinase genes was measured by RT-PCR.

encoding proteins responsible for extracellular matrix degradation.

4. Conclusions

In this study we standardized a procedure to prepare a new product (which we here call "peptide/sugar mixture") composed of aminoacids, peptides and sugars, starting from the purified cell walls of *Nicotiana* cell cultures, grown in liquid medium. The cell walls of suspension-cultured cells are known to be a rich source of extracellular glycoproteins. We provide direct evidence that a natural product can activate defense response mechanisms in both plants and animals, suggesting the existence of common signal perception mechanism and transduction pathways that regulate defense response to stresses.

Only a few compounds have been studied for their stress protective effects both in plants and on human cells, but there are no side-by-side studies with the same compound or preparation. Betaine, for example, characterized for its role in inducing defense responses in plants (Chen and Murata, 2002), was also studied by others for its protective effect on human skin cells (Warskulat et al., 2004). Proline and hydroxyproline are the two other compounds that act as important mediators of gene expression in response to stress, and treatments of either plants or human cells with these aminoacids produce relevant beneficial effects (Kiyosue et al., 1996; Krishnan et al., 2008).

In the first part of our study, we demonstrated that the cell wall extract can be used in agrochemical formulations. The use of this product as plant biostimulator has two main advantages: (i) higher activity in inducing defense response genes compared to other products already present on the market, and (ii) safe use since it is a plant-derived material. Many products used so far as biostimulators of plant defense response are mostly of animal origin (Apone et al., 2007).

The peptide/sugar mixture, shown to be effective in inducing defense mechanisms to stresses in cultured fibroblasts, indicates its potential role as an active ingredient in new cosmetic formulations as well. Besides its role in upregulating gene expression, the protective effect of the mixture was clearly confirmed by the reduction of intracellular ROS in the cells and by DNA fragmentation analysis. Moreover, its potential use in cosmetics was also enforced by its properties to enhance collagen production and stability. The experiments we showed clearly indicate that its effect on the cells is to increase pro-collagen type I and type III levels, indicating increased collagen synthesis, and/or reducing collagen degradation caused by matrix proteases.

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